FEBS Letters 582 (2008) 1613–1617

Proteins in the insulin-secreting cell line MIN6 bind the imidazoline compound BL11282

Jawed Shafqat[a], Moin Ishrat[a], Theres Jägerbrink[a], Rannar Sillard[a], Uno Mäeorg[d], Suad Efendic[b], Per-Olof Berggren[b], Sergei V. Zaitsev[b,c], Hans Jörnvall[a,*]

[a] *Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden*
[b] *The Rolf Luft Research Center for Diabetes and Endocrinology, Karolinska Institutet, Stockholm, Sweden*
[c] *Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, Russian Federation*
[d] *Institute of Chemistry, University of Tartu, Tartu, Estonia*

Received 16 March 2008; accepted 7 April 2008

Available online 15 April 2008

Edited by Robert Barouki

Abstract The imidazoline BL11282 stimulates insulin release and alters islet proteomes. Subcellular fractions of MIN6 cells showed that the membrane fraction exhibited binding to BL11282 on a Biacore chip and to BL11282-labelled magnetic beads. Bound material extracted from the beads showed a ~50 kDa differential band upon SDS–PAGE and a weaker 100 kDa band. The former was sensitive to competitive removal by preincubation of the fraction with BL11282, then highlighting the ~100 kDa band. Masspectrometric analysis revealed the ~50 kDa band to be EF1A and the ~100 kDa band to be glucose regulated P94, both of interest in insulin synthesis and secretion.

Keywords: Imidazoline compounds; Surface plasmon resonance; MIN6 cells; Elongation factor 1A; Glucose regulated protein 94

1. Introduction

A number of imidazoline compounds possess insulinotropic activity [1,2] and are of special interest in type 2 diabetes, characterized by insufficient insulin secretion from the pancreatic β-cell [3,4]. We have developed an imidazoline derivative (BL11282) which increases insulin release only at high glucose concentrations [3,5]. This is of particular interest in type 2 diabetes, since stimulation of insulin release at low or normal blood glucose levels will cause risk of hyperinsulinemia and thus resulting hypoglycemia. BL11282 has been selected as a reference compound for evaluation of other pure glucose-dependent insulinotropic compounds [6]. Therefore, the mechanisms underlying the insulin-releasing effect of this compound are of interest. Previously, we investigated proteome alterations upon exposure of rat islets to BL11282 and found that this drug has profound effects on cellular pathways in pancreatic β-cells [7].

With the positive identification of differing proteins in BL11282 exposed contra non-exposed cells established, we decided to study whether we could find BL11282 binding proteins. In the present work, we used surface plasmon resonance (SPR), a proteome extraction kit, and BL11282-labelled magnetic beads to selectively purify BL11282 binding proteins from different cellular compartments. In this manner, we found selective binding to membrane fractions and identified two binding proteins. These proteins are involved in the same functional system (protein synthesis and folding) as a number of the proteins found by proteome analysis [7] to be differentially expressed in the BL11282 treated islets. Hence, two separate analyses highlight particular protein systems of special interest.

2. Materials and methods

2.1. Materials

BL11282 was modified to give a 3-aminopropargyl derivative (Fig. 1) used for covalent attachment to prepare an affinity medium. The β-cell line MIN6 (passages 33–36) was cultured as described [8].

2.2. Protein extraction

MIN6 cells grown to 90% confluence were extracted with ProteoExtract™ subcellular proteome extraction kit (Calbiochem) into four fractions (cytosolic, membranes, nuclear and matrix). Those cells which were detached from the plate during the extraction procedure were extracted as suspended cells according to the manufacturer's protocol. The fractions obtained were frozen at −20 °C in aliquots and were used for analysis within a month. BL11282 binding activity in the fractions was determined by SPR using a Biacore 3000 instrument (GE Healthcare).

2.3. Surface plasmon resonance (SPR) measurements

Binding activities of sub-cellular fractions were determined in a Biacore 3000 instrument. The modified BL11282 compound was immobilized on a CM5 sensor chip surface by amine coupling. In brief the chip surface was first washed for 1 min with 4 M LiCl and 0.2% surfactant P20 (SP20) (Biacore) in Tris buffered saline (TBS). Two lanes on the chip surface were activated according to the standard procedure with an injection of 0.05 M *N*-hydroxysuccinimide (NHS)/0.2 M *N*-ethyl-*N*′-[3-dimethylamino)propyl]carbodiimide (EDC) HCl for 7 min at 5 μl/min. The modified BL11282 compound (40 μg/ml in 10 mM sodium acetate, pH 5) was immobilized on lane 2 at a flow of 5 μl/min. After immobilization both lanes were subjected to 1 M ethanolamine to deactivate remaining activated carboxylic groups. Lane 1, only activated and deactivated (without addition of modified BL11282), was used as control in the binding experiments. TBS containing 0.005% SP20 was used as running buffer, and different fractions were injected at 10 μl/min for 2 min. The surface was regenerated after each cycle with 6 M guanidine–HCl, pH 6.8, and frequently treated also with diluted trypsin in 20 mM ammonium bicarbonate, pH 8, to remove proteins non-specifically adsorbed onto the chip surface.

*Corresponding author. Fax: +46 8 33 74 62.
E-mail address: Hans.Jornvall@ki.se (H. Jörnvall).

0014-5793/$34.00
doi:10.1016/j.febslet.2008.04.007

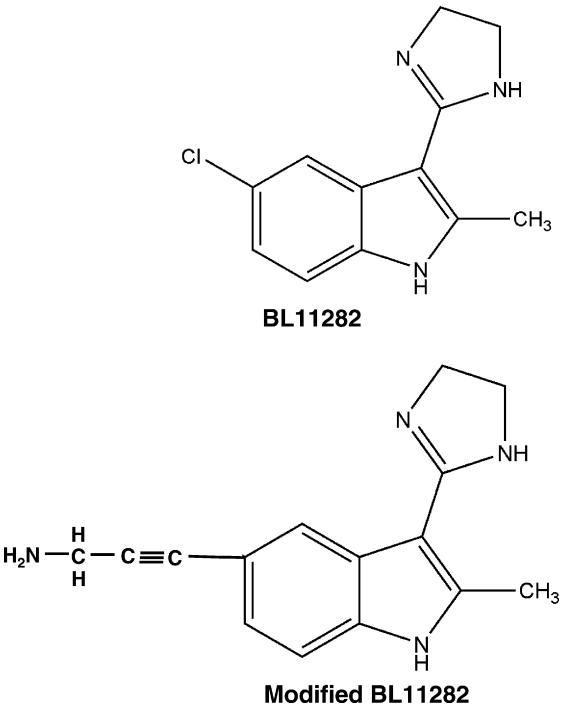

Fig. 1. Structures of BL11282 and the modified derivative used for the labelling of the chips and magnetic beads.

2.4. Binding experiments using beads

For recovery of the BL11282 binding protein(s), magnetic Dynabeads M-270 (Dynal Beads, Invitrogen) were activated with EDC/NHS in a manner similar to that for the Biacore chips. In brief, the beads were washed twice with 25 mM MES buffer, pH 5, after which freshly dissolved EDC and NHS in MES buffer were added for 30 min at room temperature. Excess EDC/NHS was removed by washing twice with MES buffer. Subsequently, the activated beads were incubated with the modified BL11282 (40 μg/ml) for 30 min at room temperature during rotation. After incubation the unbound compound was removed by washing twice for 15 min with TBS. Remaining activated carboxylic groups on the beads were blocked by incubation with 1 M ethanolamine for 30 min, followed by washing with TBS for 3×15 min. For control experiments, beads activated with EDC/NHS and deactivated with ethanolamine were used.

Membrane fractions prepared from MIN6 cells were incubated with both labelled and unlabelled (control) beads for 4 h at room temperature. After incubation the beads were settled with the help of a magnet, and the supernatant was transferred to a fresh tube. The beads were then washed once with extraction buffer (F2) and twice with TBS for 15 min, and all supernatants were discarded. Bound material on the beads was extracted, first with 1 M NaCl for 15 min and then with 50 mM NaOH for 30 min at room temperature with rotation. In both steps the beads were settled with a magnet and the supernatants collected. Finally, the beads were boiled in $1 \times$ SDS–PAGE sample buffer to extract remaining bound proteins. All fractions (obtained with NaCl, NaOH, and boiling) were analyzed on Bis-Tris SDS/PAGE and silver-stained with Silverquest Kit (Invitrogen).

Competition experiments using Biacore and labelled beads were carried out by pre-incubation of the membrane fraction with 0–5 mM of free BL11282 for 1 h at room temperature.

2.5. In-gel digestion and mass spectrometry

The protein bands from the gels were excised manually and in-gel digested with trypsin using a MassPrep robotic system (Waters) [9]. The digests were concentrated under a stream of nitrogen and analyzed on a Q-TOF Ultima API mass spectrometer (Waters) coupled on-line with CapLC (Waters). The mass spectrometer was operated in positive ion mode employing data-dependent acquisition over a mass range of 350–2000 *m/z*. The sample was separated on a Waters Atlantis C18 column (3 μm, 100 Å, 75 μm × 150 mm) with a solvent system of 5% acetonitrile/0.1% formic acid in water (solvent A) and 95% acetonitrile/0.1% formic acid (solvent B) after desalting with a LC Packings Nanoease Trapcolumn (300 μm ID × 1 mm; 5% acetonitrile/0.1% formic acid; 20 μl/min).The peptides were eluted with a linear gradient of 10–50% solvent B for 20 min at 200 nl/min. Data analysis was performed using ProteinLynx Global SERVER 2.1 (PLGS 2.1, Waters) software and MassLynx peptide sequence software version 4.0 (Waters). Data sets were analyzed using the NCBI BLAST and Mascot search engines against Swissprot with the genomic database of *Mus musculus* chosen for both search methods without constrictions regarding molecular weight or pI. Mass spectra and sequences were manually examined and confirmed to be of sufficient quality for confident identification. Only sequences of at least eight residues were taken into consideration and an Expect value $\leqslant 0.05$ for at least one sequenced peptide was required for identification.

3. Results

3.1. Cellular fractionation and SPR analysis

BL11282 binding activity in different fractions of MIN6 cells was determined by SPR using a Biacore 3000 instrument. The cells were fractionated into four parts, cytosolic, membrane, nuclear and matrix, with a subcellular proteome extraction kit, and binding activities were monitored for each fraction (cf. [10]). Binding experiments were performed after fractionation and then again after incubation of labelled beads or control beads (see below). Most of the binding activity was found in the membrane fraction, while some was also detectable in the cytosolic fraction. Although absolute binding varied between different extractions, the membrane fraction was always the one with higher binding than the cytosolic fraction. The net binding (control lane extracted) was typically around 1000 response units for the membrane fraction.

3.2. Binding experiments

For the identification of binding proteins, BL11282-labelled magnetic beads were employed. After incubation, supernatants were recovered and monitored for binding in the Biacore instrument. Fig. 2 shows a considerable loss of binding in the supernatant of labelled beads, whereas only a little decrease is observed in the supernatant of the control beads. Material that was giving rise to the binding response in the BL11282-labelled lane of the SPR-chip is now apparently attached to the

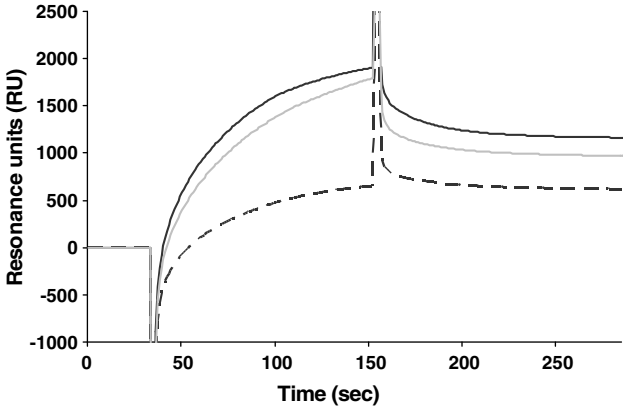

Fig. 2. SPR analysis of binding activity in the membrane fraction of MIN6 cells (black line), after incubation with unlabelled (control) beads (gray line) and after incubation with BL11282-labelled beads (dashed line). All curves are after blank subtraction.

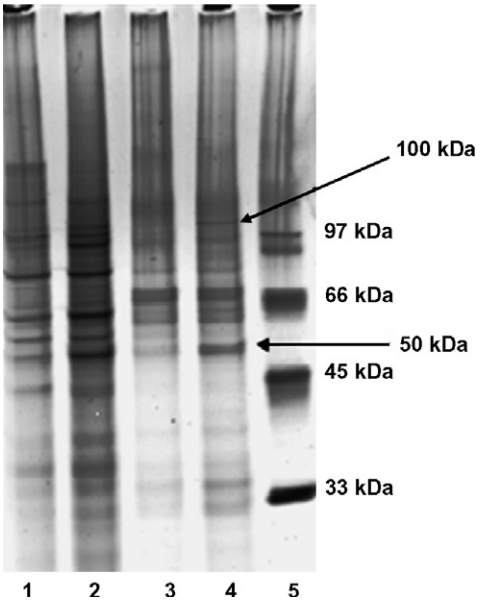

Fig. 3. SDS–PAGE of the fractions extracted from the beads. 50 mM NaOH extract of the unlabelled (lane 1) and labelled (lane 2) beads, boiled extract of the unlabelled (lane 3), and labelled (lane 4) beads, marker proteins (lane 5).

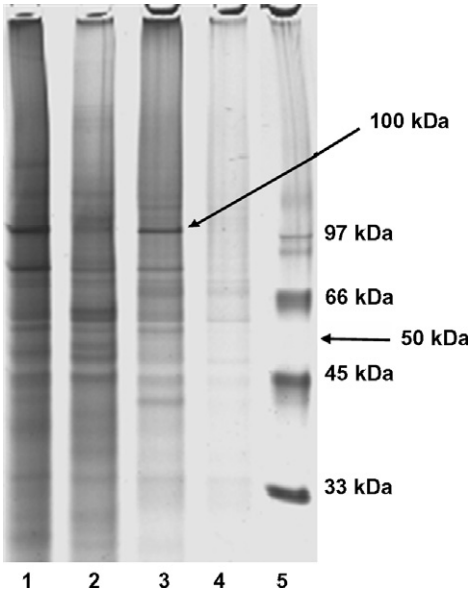

Fig. 4. SDS–PAGE of the fractions obtained after competition experiments (pre-incubation with 1 mM BL11282) before incubation with labelled or unlabelled beads. 50 mM NaOH extract of the labelled (lane 1) and unlabelled (lane 2) beads, boiled extract of the labelled (lane 3), and unlabelled (lane 4) beads, marker proteins (lane 5).

labelled beads. The beads, extracted with NaCl, NaOH, and by boiling, showed various bands on SDS–PAGE but a strong band of about 50 kDa was specifically present in different MIN6 cell preparations (Fig. 3). The presence of a weak ~100 kDa band was also observed (Fig. 3). These bands were identified by peptide masspectrometric analysis after digestion (below).

3.3. Competition experiments

Competition experiments were performed by pre-incubation of the membrane fraction with free BL11282. In Biacore analysis, the presence of the free compound failed to compete with the binding, while the bead extracts, after competition with 1 mM free compound showed removal of the ~50 kDa band, resulting in the ~100 kDa band as the strongest one seen (Fig. 4).

3.4. Mass spectrometric identifications

The tryptic peptides of the two protein bands identified in the binding experiments were analyzed by LC–MS/MS. The 50 kDa band was identified as elongation factor 1 alpha (eEF1A) by recovery of the sequence of three peptides, and the 100 kDa band was identified by the Mascot search engine (significance threshold $p = 0.05$) as glucose regulated protein 94 (GRP94). Two peptide sequences and an additional mass corresponding to a third peptide were recovered from the 100 kDa band (Table 1).

4. Discussion

Using sub-cellular fractionation, SPR-binding studies and affinity purification on BL11282-labelled beads, we have identified two binding proteins in a mouse insulinoma cell line. Extracts from the cell line showed the presence of clear binding to BL11282-labelled SPR-chips, which disappeared upon incubation with BL11282-labelled beads. In competition binding experiments using SPR measurements, the presence of free compound (in concentration up to 1 mM) did not compete, presumably because the BL11282 derivative on the Biacore chips is highly available. However, in competition experiments

Table 1
Identification of protein bands by MS/MS sequence analysis of tryptic peptides

Protein	Accession number (swissprot)	Peptide sequence	Position	Expect value
eEF1A-1/2[a] (50 kDa)	P10126	LPLQDVYK	248–255	0.03
	P62631	IGGIGTVPVGR	256–266	0.083
		QTVAVGVIK	431–439	0.026
GRP94 (100 kDa)	P08113	TDDEVVQR	44–51	1.0
		ELISNASDALDK	103–114	0.007
		EVEEDEYK[b]	349–356	4.3

[a]Variants 1 and 2 of eEF1A differ at positions outside those now analyzed.
[b]Identified by mass only (too weak for sequence analysis).

using beads, after pre-incubation of the membrane fraction with 1 mM free compound, the eEF1A band was competitively removed and the ~100 kDa GRP94 band then emerged as the strongest band (Fig. 4). The appearance of the GRP94 band in the bead experiments after competitive removal of eEF1A shows that GRP94 has a different mode of binding than eEF1A with BL11282. In a previous report we have studied proteomes of rat pancreatic islets by a 2-D gel approach and did then not find eEF1A and GRP94 [7]. However, because of the low abundance of these proteins even in the present affinity-enriched preparations (Fig. 3), their absence of detection in overall proteome 2-D gel comparisons is not surprising.

In type 2 diabetes the inadequate insulin release requires an extra stimulus to secrete more insulin which is usually accomplished by the sulfonylurea drugs but with a risk of severe hypoglycemia [11]. BL11282, unlike sulfonylurea, stimulates glucose dependent insulin release and not insulin release at basal glucose levels, thus the risk of hypoglycemia is reduced [3,5]. A pure glucose-dependent insulinotropic activity of BL11282 involves the K_{ATP} channel independent pathways of stimulation of insulin secretion, the nature of which are still not fully understood. The identifications of eEF1A and GRP94 as BL11282 binding proteins, point to possible mechanisms involved in BL11282-stimulated insulin secretion.

EF1A has a major function in delivery of aminoacyl-tRNA to the ribosome but it also possesses many other functions like stabilization of the microtubules in a Ca^{2+}/calmodulin-dependent manner [12] and in the regulation of the actin cytoskeleton and cell morphology [13]. It has been reported that biosynthesis of insulin is controlled at the translational level [14] and the translation of mRNA encoding insulin is preferentially increased in pancreatic β-cells by glucose stimulation [15]. In a recent report control of mRNA translation through phosphorylation of eukaryotic initiation factor 2 (eIF2α) has been found essential for the increased insulin production [16]. It has also been demonstrated that EF1A is a potential associated binding partner for Akt/PKB [17], a protein kinase involved in regulation of insulin secretion [18].

GRP94 is a molecular chaperone in the endoplasmic reticulum (ER) where it is known to bind ATP, peptides [19] and Ca^{2+} [20]. Its role in maturation and transport of thyroglobulin along the secretory pathway has been reported [21]. Although a role of GRP94 in the synthesis of insulin is not firmly established, maturation of proinsulin in the ER of the β-cells may well require GRP94 [22], and a decrease in GRP94 is accompanied by a loss of glucose-responsiveness in insulin-secreting cells [23].

In conclusion, our results suggest that BL11282 binds to elongation factor 1 alpha and to GRP94 in insulin-secreting cells, both proteins of interest in insulin synthesis and secretion.

Acknowledgements: We are grateful to Carina Palmberg and Gunvor Alvelius for skilful assistance. This work was supported by funds from the Swedish Research Council (Project 03X-3532), Karolinska Institutet, the Novo-Nordisk Foundation, the Swedish Diabetes Association, Knut and Alice Wallenberg Foundation, The Family Erling-Persson Foundation, and Berth von Kantzow's Foundation.

References

[1] Schulz, A. and Hasselblatt, A. (1989) An insulin releasing property of imidazoline derivatives is not limited to the compounds that block α-adrenoceptors. Naunyn Schmiedebergs Arch. Pharmacol. 340, 321–327.

[2] Zaitsev, S.V., Efanov, A.M., Efanova, I.B., Larsson, O., Östenson, C.G., Gold, G., Berggren, P.-O. and Efendic, S. (1996) Imidazoline compounds stimulate insulin release by inhibition of K(ATP) channels and interaction with the exocytotic machinery. Diabetes 45, 1610–1618.

[3] Efendic, S., Efanov, A.M., Berggren, P.-O. and Zaitsev, S.V. (2002) Two generations of insulinotropic imidazoline compounds. Diabetes 51 (Suppl. 3), S448–S454.

[4] Efanov, A.M., Appelskog, I.B., Abdel-Halim, S.M., Khan, A., Bränström, R., Larsson, O., Östenson, C.G., Mest, H.J., Berggren, P.-O., Efendic, S. and Zaitsev, S.V. (2002) Insulinotropic activity of the imidazoline derivative RX871024 in the diabetic GK rat. Am. J. Physiol. Endocrinol. Metab. 282, E117–E124.

[5] Efanov, A.M., Zaitsev, S.V., Mest, H.J., Raap, A., Appelskog, I.B., Larsson, O., Berggren, P.-O. and Efendic, S. (2001) The novel imidazoline compound BL11282 potentiates glucose-induced insulin secretion in pancreatic beta-cells in the absence of modulation of K(ATP) channel activity. Diabetes 50, 797–802.

[6] Bahekar, R.H., Jain, M.R., Goel, A., Patel, D.N., Prajapati, V.M., Gupta, A.A., Jadav, P.A. and Patel, P.R. (2007) Design, synthesis, and biological evaluation of substituted-*N*-(thieno[2,3-b]pyridin-3-yl)-guanidines, *N*-(1H-pyrrolo[2,3-b]pyridin-3-yl)-guanidines, and *N*-(1H-indol-3-yl)-guanidines. Bioorg. Med. Chem. 15, 3248–3265.

[7] Jägerbrink, T., Lexander, H., Palmberg, C., Shafqat, J., Sharoyko, V., Berggren, P.-O., Efendic, S., Zaitsev, S. and Jörnvall, H. (2007) Different protein expression in pancreatic islets after treatment with an imidazoline compound. Cell. Mol. Life Sci. 64, 1310–1316.

[8] Sharoyko, V.V., Zaitseva, I.I., Varsanyi, M., Portwood, N., Leibiger, B., Leibiger, I., Berggren, P.-O., Edendic, S. and Zaitsev, S.V. (2005) Monomeric G-protein, Rhes, is not an imidazoline-regulated protein in pancreatic beta-cells. Biochem. Biophys. Res. Commun. 338, 1455–1459.

[9] Roblick, U.J., Hirschberg, D., Habermann, J.K., Palmberg, C., Becker, S., Kruger, S., Gustafsson, M., Bruch, H.P., Franzén, B., Ried, T., Bergman, T., Auer, G. and Jörnvall, H. (2004) Sequential proteome alterations during genesis and progression of colon cancer. Cell. Mol. Life. Sci. 61, 1246–1255.

[10] Lindahl, E., Nyman, U., Melles, E., Sigmundsson, K., Ståhlberg, M., Wahren, J., Öbrink, B., Joseph, B., Shafqat, J. and Jörnvall, H. (2006) Cellular internalization of proinsulin C-peptide. Cell. Mol. Life Sci. 64, 479–486.

[11] Krentz, A.J. and Bailey, C.J. (2005) Oral antidiabetic agents: current role in type 2 diabetes mellitus. Drugs 65, 385–411.

[12] Moore, R.C., Durso, N.A. and Cyr, R.J. (1998) Elongation factor-1alpha stabilizes microtubules in a calcium/calmodulin-dependent manner. Cell. Motil. Cytoskeleton 41, 168–180.

[13] Gross, S.R. and Kinzy, T.G. (2005) Translation elongation factor 1A is essential for regulation of the actin cytoskeleton and cell morphology. Nat. Struct. Mol. Biol. 12, 772–778.

[14] Itoh, N. and Okamoto, H. (1980) Translational control of proinsulin synthesis by glucose. Nature 283, 100–102.

[15] Schuit, F.C., In't Veld, P.A. and Pipeleers, D.G. (1988) Glucose stimulates proinsulin biosynthesis by a dose-dependent recruitment of pancreatic beta cells. Proc. Natl. Acad. Sci. USA 85, 3865–3869.

[16] Scheuner, D., Mierde, D.V., Song, B., Flamez, D., Creemers, J.W., Tsukamoto, K., Ribick, M., Schuit, F.C. and Kaufman, R.J. (2005) Control of mRNA translation preserves endoplasmic reticulum function in beta cells and maintains glucose homeostasis. Nat. Med. 11, 757–764.

[17] Lau, J., Castelli, L.A., Lin, E.C. and Macaulay, S.L. (2006) Identification of elongation factor 1α as a potential associated binding partner for Akt2. Mol. Cell. Biochem. 286, 17–22.

[18] Bernal-Mizrachi, E., Fatrai, S., Johnson, J.D., Ohsugi, M., Otani, K., Han, Z., Polonsky, K.S. and Permutt, M.A. (2004) Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced Akt activity in pancreatic islet beta cells. J. Clin. Invest. 114, 928–936.

[19] Booth, C. and Koch, G.L.E. (1989) Perturbation of cellular calcium induces secretion of luminal ER proteins. Cell 59, 729–737.

[20] Csermely, P., Schnaider, T., Soti, C., Prohaszka, Z. and Nardai, G. (1998) The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacol. Ther. 79, 129–168.
[21] Muresan, Z. and Arvan, P. (1997) Thyroglobulin transport along the secretory pathway: investigation of the role of molecular chaperone, GRP94, in protein export from the endoplasmic reticulum. J. Biol. Chem. 272, 26095–26102.
[22] Kitiphongspattana, K., Mathews, C.E., Leiter, E.H. and Gaskins, H.R. (2005) Proteasome inhibition alters glucose-stimulated (pro)insulin secretion and turnover in pancreatic {beta}-cells. J. Biol. Chem. 280, 15727–15734.
[23] Dowling, P., O'Driscoll, L., O'Sullivan, F., Dowd, A., Henry, M., Jeppesen, P.B., Meleady, P. and Clynes, M. (2006) Proteomic screening of glucose-responsive and glucose non-responsive MIN-6 beta cells reveals differential expression of proteins involved in protein folding, secretion and oxidative stress. Proteomics 6, 6578–6587.